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Early intervention with Interleukin-1 Receptor Antagonist Protein modulates catabolic microRNA and mRNA expression in cartilage after impact injury

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SUMMARY

Objective: The purpose of this controlled laboratory study was to determine the efficacy of Interleukin-1 Receptor Antagonist Protein (IRAP) treatment as an early intervention strategy by examining the changes in microRNA and mRNA expression in cartilage in an *ex-vivo* porcine knee joint impact model.
Methods: Custom impact device was used to create replicable injury *ex-vivo* to intact porcine knee joint. Injury was caused by dropping a 10 kg weight one time from 1 m directly above the knee in extension. One hour after impact 20 μg/ml IRAP solution was intra-articularly injected. At 8 h post-injury, cartilage samples were harvested for cell viability and genetic expression analysis. Genetic expression of miR-27b, miR-140, miR-125b, ADAMTS-4, ADAMTS-5, MMP-3, IL-1β, and TNF-α were analyzed by RT-PCR. Cell viability image analysis was performed using ImageJ software. Groups were compared by analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. A *P*-value <0.05 was considered significant.
Results: At 8 h after IRAP treatment, expressions of ADAMTS-4, ADAMTS-5, MMP-3, IL-1β, and TNF-α in cartilage were significantly down-regulated from injury group (all $P < 0.001$). MiR-140, miR-125b, and miR-27b expressions were significantly up-regulated after treatment as compared to control and injury groups (all $P < 0.001$).
Conclusion: This study demonstrates that IRAP treatment administered during acute phase of cartilage impact injury increases expression levels of miR-140, miR-125b, and miR-27b in cartilage, indicating increased inhibition of their respective matrix-degrading enzymes. Clinically, these findings support the potential of IRAP treatment as an early intervention strategy for the prevention of cartilage degeneration after impact injury.

Introduction

Traumatic injury to the knee has been shown to lead to Post-Traumatic Osteoarthritis (PTOA)[1]. Cartilage degeneration can occur within 15 years of a traumatic knee injury; therefore, PTOA has become more prevalent in young individuals and is responsible for up to 12% of OA cases[2]. Current therapeutic strategies for PTOA, such as rehabilitation, molecular-based injections, total joint replacements, etc., are most often administered after the injured cartilage has begun to degenerate and cause disabling pain to the patient. At this point, the therapies are merely to assuage pain and are unable to reverse the permanent cartilage damage. Unlike primary degenerative OA, an exact time of injury can be correlated to the initiation of the disease and presents the opportunity for intervention to prevent PTOA development.

After a traumatic knee injury, with or without cartilage fracture, inflammatory cytokine concentration increases in the synovial fluid and injured tissues[3]. The primary cytokines produced in an inflammatory environment are Interleukin (IL)-1β and Tumor Necrosis Factor (TNF)-α[3,4]. Up-regulation of IL-1β after injury triggers a catabolic pathway, disrupting cellular homeostasis by increasing

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aggrecanase and collagenase production and decreasing collagen and aggrecan production[5]. This imbalance of anabolic and catabolic processes can be traced back to aberrant epigenetic regulation by microRNAs (miRNAs), ~20-nucleotide non-coding RNAs[6]. In their simplest form, miRNAs negatively regulate target messenger RNA (mRNA) post-transcriptionally by promoting degradation, deadenylation, or repression of translation[7]. Three miRNAs miR-125b, miR-140 and miR-27b, among others, have been implicated in the progression of OA and their expressions have been shown to be influenced by an inflammatory environment[8–10]. MiR-125b and miR-140 have been identified as regulators of aggrecanase-1 (A Disintegrin And Metalloproteinase with Thrombospondin Motifs [ADAMTS]-4)[8] and aggrecanase-2 (ADAMTS-5)[9], respectively, and are expressed significantly lower in OA and IL-1β-treated chondrocytes than in normal chondrocytes. MiR-27b directly regulates Matrix Metalloproteinase (MMP)-13 and its expression is significantly decreased in OA chondrocytes and is regulated by the IL-1β-activated catabolic pathway[10]. Additionally, MMP-3, which cleaves proteoglycans and is highly expressed in OA cartilage, activates pro-MMP-13, furthering matrix breakdown[11]. Proper regulation of aggrecanases and collagenases is critical to achieve homeostasis in cartilage after injury. Thus, there is a substantial need for a method of early intervention that will effectively prevent or delay the degeneration of cartilage after acute traumatic injury.

IL-1 Receptor Antagonist Protein (IRAP), naturally occurring in the body, effectively binds to the IL-1 receptor on the chondrocyte membrane and blocks IL-1α/β from triggering its catabolic pathway[12]. Preclinical and clinical studies[5,12–14] have demonstrated the efficacy of IRAP in reducing catabolic activities and joint discomfort in models of established OA. Recent studies using a mouse articular cartilage fracture model have shown that IRAP, as an early intervention treatment, significantly reduced cartilage degeneration and synovial inflammation[15,16]. However, how IRAP treatment affects catabolic genetic regulation in cartilage during the acute phase of impact injury has not yet been elucidated.

Therefore, the objective of this controlled laboratory study was to determine the efficacy of IRAP treatment as an early intervention strategy by examining the changes in miRNA and mRNA expression in cartilage after impact injury. Our hypothesis was that early intervention with IRAP during the acute phase of cartilage injury can modulate catabolic and inflammatory gene expressions caused by impact by altering the expressions of miRNA.

Methods and materials

Injury model development

A custom impact device was developed to create replicable injury *ex-vivo* to intact porcine knee joint. Porcine legs from 35 to 40 kg pigs were acquired from the University of Miami Department of Veterinary Resources Tissue Sharing program (Institutional Animal Care and Use Committee approved source) and were disarticulated at the hip within 2 h of death. Skin and muscle surrounding both joints were removed to fit the leg properly in extension in a confined chamber of the impact device, while maintaining the synovial membrane and joint capsule intact. The confined chamber prevented knee flexion upon impact. An impact cap comprised of a polycarbonate tube and metal plate was placed securely over the femoral head to ensure flat impact surface. A knee joint from each pig was randomly selected and subjected to an impact force about 4.6 kN by dropping a 10 kg weight one time from 1 m directly above the knee in extension. The impact force was chosen based on average mass withstood per leg (40 kg pig/4 legs = 10 kg/leg). The chosen impact force did not result in visible cartilage damage or bone fracture. A force transducer (Omega DLC101 Impact Sensor, Omega Engineering Inc.) was placed below the platform on which the leg stood to assure impact force reproducibility. The contralateral joint of the same pig (control) was placed within chamber of the impact device but was not subjected to impact. Immediately after impact, limbs were wrapped in saline-soaked gauze to prevent dehydration and placed in humidified chamber at physiological temperature 37°C and 20% oxygen until time point. At 1 h post-impact, control and injury (impact without treatment) joints received 1 mL of sterile Phosphate Buffered Saline (PBS) injections to simulate intra-articular injection.

To determine appropriate end time point for genetic changes to take place, cartilage samples from impacted areas on femoral condyles of control and injury knees were harvested at 3 and 8 h post-impact, flash-frozen, and stored at −80°C for genetic expression analysis. MiR-140, miR-125b, miR-27b, IL-1β, TNF-α, MMP-3, ADAMTS-4, and ADAMTS-5 were selected as indicators of injury for model development. Meniscus samples from impacted region were also harvested and stored for genetic expression analysis for subsequent treatment study.

Fuji film pressure and area analysis

A medium-grade pressure sensitive film (FujiFilm; Sensor Products, Inc. Madison, NJ) was used to determine the contact area and pressure created by the 10 kg impact, as previously described by Clark *et al.*[17]. In brief, the patellofemoral tendon and synovial membrane were dissected to allow access into the knee joint. The film was sealed between thin polyethylene adhesive layers, and two strips of 2×6 cm^2 were inserted into the femoral-tibial joint in an anterior/posterior direction, separated by the anterior and posterior cruciate ligaments. After impact, digital images of the Fujifilm stain were obtained at a resolution of 600 ppi using a digital scanner. To verify the linear relationship between applied pressure and stain intensity, Fujifilm strips underwent a series of four static load tests. Compressive loads, ranging from 15 to 40 MPa, were applied by a 3.36 mm diameter cylindrical indentor using an Instron materials-testing machine (Instron Co., Norwood, MA).

Each Fujifilm stain image underwent initial processing to account for the granular texture of the film (MATLAB 2014a)[18]. Images were divided into 4×4 pixel areas. The mean intensity of the pixels in the area was then applied across the entire 4×4 pixel area. Pressures calculated from standard curve were applied to impacted stains to produce a pressure distribution map. Injured area defined by pressures >20 MPa were then calculated. Multiple studies reported impact stresses greater than 20 MPa led to chondrocyte death, collagen matrix rupture, and cartilage degeneration[19–24].

Immunohistochemical analysis

Cartilage samples from impacted areas on control and injured joints were placed in 10% neutral buffered formalin for 24 h. The samples were paraffin embedded, and sectioned cross-sectionally (5 μm) for staining. Immunohistochemistry was used to localize IL-1β and TNF-α cytokine proteins within the cartilage tissue. Endogenous peroxidase was quenched for 30 min in the dark with 0.3% H_2O_2 in distilled water. After rinsing with distilled water and PBS, 10% normal horse serum was used to block nonspecific background. Sections were stained with a rabbit polyclonal antibody against human IL-1β (anti-human IL-1β (H-153), sc-7884; Santa Cruz Biotechnology, Inc) or human TNF-α (anti-human TNF-α (H-156), sc-8301; Santa Cruz Biotechnology, Inc) at 1:50 dilution at 4°C for 18 h. Negative staining control sections were incubated with antibody diluent without primary antibody and then carried through the same secondary antibody and detection protocols as the positive control sections. Chromogenic detection was achieved

with diaminobenzidine (DAB) substrate (Vectastain), followed by counterstaining with hematoxylin. Sections were observed and digitally photographed at 200× magnification.

IRAP administration

For IRAP treatment, the procedure described previously was used to injure the joint. One hour after impact, 20 μg of recombinant human IRAP (PeproTech; Rocky Hill, NJ) in 1 mL of PBS was administered to injured knee *via* intra-articular injection. The dose (20 μg/mL) was calculated from successful reports of inhibition of catabolic gene expression with IRAP in porcine knee tissues *in vitro*[13]. The contralateral joint of the same pig served as the control in the same manner previously described. At 8 h after injury, samples from impacted region on articular cartilage on femoral condyles and meniscus from both groups were harvested, flash-frozen, and stored at −80°C for analyses of miRNA and mRNA expressions.

Analysis of chondrocyte viability

Cartilage samples from control, injury, and treatment joints were stained for cell viability at time of harvest. In brief, a 100-micron thick full-depth sample was prepared and incubated in PBS containing 1 μmol/L ethidium homodimer-1 and 1 μmol/L calcein AM from the Live/Dead Viability/Cytotoxicity Kit (Invitrogen). Staining was visualized on an inverted fluorescent microscope with 495 nm/515 nm excitation/emission for calcein (live cells) and 495 nm/635 nm excitation/emission for ethidium homodimer (dead cells).

Image analysis was conducted to determine the number of live and dead cells, as described in our previous study[24]. A defined region of interest was identified (1.11 mm wide × 0.88 mm deep at the center of the sample, measured from the surface down) from images taken through a 10× objective lens utilizing MATLAB R2014a (MathWorks Natick, MA). The resulting images were analyzed with ImageJ software, and percent cell viability (number of live cells × 100/total number of cells) was calculated.

Analyses of mRNA and miRNA expressions

Cartilage tissue adjacent to the areas used for cell viability staining was processed for analyses. For meniscus, tissue samples from impacted region were used for analyses. Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform extraction method[25]. For mRNA expression analyses, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize cDNA from 0.25 μg total RNA following manufacturer's instructions. For miRNA expression analyses, the qScript microRNA cDNA synthesis kit (Quanta Biosciences) was used to polyadenylate miRNAs and synthesize cDNA from 0.25 μg total RNA following manufacturer's instructions. Expression of selected mRNA and miRNA was analyzed using StepOnePlus (Applied Biosystems) quantitative real-time PCR system. Expression of ribosomal 18S and RNU6B were used as endogenous controls for mRNA and miRNA, respectively. Primer sequences are listed in Table I. Quantification of expression levels was determined by the $2^{-\Delta\Delta Ct}$ method and normalized to expression levels of respective control leg.

Statistical analysis

In this study, we analyzed 3 h control joints ($n = 3$), 8 h control joints ($n = 10$), 3 h injury joints ($n = 3$), 8 h injury joints ($n = 5$), and 8 h treatment joints ($n = 5$). From each joint, four tissue samples each were harvested from the impacted regions of the femoral condyle and meniscus. The samples from each joint were then averaged prior to statistical analysis. In model development, comparisons between injured area and maximum stress on the medial and lateral condyles and between control and injury groups at 3 and 8 h were performed by two-sample two-tailed Student's *t*-test. For statistical analysis of IRAP treatment, comparison of control, injury, and treatment groups at 8 h post-injury was performed by a one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test (MATLAB R2014a). For all tests, a *P*-value <0.05 was considered statistically significant.

Results

Model development

The average impact load was 4.7 ± 1.6 kN ($n = 8$) as measured by the impact force sensor below the platform. After creating a standard curve (Fig. 1), pressure distribution analysis (Fig. 2) showed an average combined injured area of 23.5 ± 1.9 mm^2 ($n = 8$) greater than 20 MPa on both medial and lateral condyles with the lateral and medial condyles having roughly equal injured areas (lateral: 9.6 ± 2.7 mm^2, medial: 8.4 ± 3.2 mm^2, $P = 0.75$). In addition, there was no significant difference between maximum pressure recorded on medial (25.1 ± 1.9 MPa) and lateral (25.5 ± 1.1 MPa) condyles after impact ($P = 0.64$).

Analyses of mRNA and miRNA expression in cartilage after injury showed an increase in ADAMTS-4 ($P = 0.0001$) and MMP-3 ($P = 0.001$) at 8 h and ADAMTS-5 ($P = 0.05$, 0.001) expression at 3 and 8 h, respectively [Fig. 3(A)]. IL-1β and TNF-α were significantly up-regulated at 3 and 8 h post-impact, maintaining similar levels of up-regulation across both time points ($P < 0.01$). Increased IL-1β and TNF-α expression in injured cartilage 8 h post-impact was verified by immunohistochemical staining (Fig. 4). At 3 h post-injury, miR-140 and miR-125b were significantly up-regulated (both $P < 0.05$), but at 8 h post-injury, miR-140 and miR-125b were significantly down-regulated ($P = 0.03$, 0.04 respectively) [Fig. 3(B)].

Effect of IRAP treatment on cell viability and gene expression

At 8 h post-injury and treatment, chondrocyte viability significantly decreased in injury group as compared to control (62.3 ± 5.9% vs 82.2 ± 2.5%, $P = 0.013$) (Fig. 5). There were no significant differences found between control and treatment (82.2 ± 2.5% vs 74.7 ± 3.2%, $P = 0.42$) nor between injury and treatment ($P = 0.12$).

Significant effect of treatment at 8 h post-injury was shown for aggrecanase and inflammatory cytokine expressions [Fig. 6(A)]. Expressions of ADAMTS-4, ADAMTS-5, MMP-3, IL-1β, and TNF-α in cartilage were significantly lower than injury group expressions (all $P < 0.001$). IRAP treatment after injury also significantly affected cartilage miRNA expressions [Fig. 6(B)]. Expressions of miR-140, miR-125b, and miR-27b were significantly up-regulated after treatment as compared to control and injury groups (all $P < 0.001$).

To determine the metabolic effect of IRAP on cartilage, we examined the relative expression of aggrecan and collagen II [Fig. 6(A)]. At 8 h post-impact, aggrecan expression was significantly increased ($P = 0.001$) as compared to controls. After injury and treatment, aggrecan expression was not significantly different from the control or injury groups ($P = 0.18$ and 0.26, respectively). No significant difference in collagen II expression was observed between groups (ANOVA $P = 0.377$) (data not shown).

Because this model allows for simultaneous treatment of intra-articular tissues, we examined the mRNA and miRNA expressions in meniscus 8 h after injury and IRAP treatment [Fig. 7(A) and (B)].

Table I
Quantitative real-time PCR primers

Gene	Primer sequences	Reference/ GenBank/ miRbase Accession#
ADAMTS-4	F:5′-AGGAGGAGATCGTGTTTCCAGAGA-3′ R:5′-AAAGGCTGGCAAGCGGTACAACAA-3′	[26]
ADAMTS-5	F:5′-TTCGACATCAAGCCATGGCAACTG-3′ R:5′-AAGATTTACCATTAGCCGGGCAGG-3′	[26]
IL-1β	F:5′-AATGCAACAGGGTGTGGGC-3′ R:5′-CCACACCAGAAGTGCATTTG-3′	X74568.1
TNF-α	F:5′-CCCTGTGAGGGGGCAGGACA-3′ R:5′-ACCCAAGGACCCCAGCGAGT-3′	X54859.1
MMP-3	F:5′-TCCTGATGTTGGTTACTTCAGCAC-3′ R:5′-TTGACAATCCTGTAAGTGAGGTCATT-3′	[27]
Aggrecan (AGN)	F:5′-AGACAGTGACCTGGCCTGAC-3′ R:5′-CCAGGGGCAAATGTAAAGG-3′	[28]
Collagen II (COL2)	F:5′-TGAGAGGTCTTCCTGGCAAA-3′ R:5′-ATCACCTGGTTTCCCACCTT-3′	[28]
18S	F:5′-CGGCTACCACATCCAAGGA-3′ R:5′-AGCTGGAATTACCGCGGCT-3′	[26]
hsa-miR-140-5p	5′-AGTGGTTTTACCCTATGGTAG-3′	MIMAT0000431
ssc-miR-125b-5p	5′-TCCCTGAGACCCTAACTTGTGA-3′	MIMAT0000423
hsa-miR-27b-5p	5′-AGAGCTTAGCTGATTGGTGAACA-3′	MIMAT0004588
RNU6B	F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′ R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′	[29]

After injury, expressions of ADAMTS-4, IL-1β, MMP-3, and TNF-α were significantly up-regulated in meniscus tissue ($P = 0.03$, 0.001, 0.005, and 0.001, respectively). ADAMTS-5, while not significant, consistently tended towards up-regulation in the injury group ($P = 0.09$). Expression of miR-27b after injury was significantly decreased as compared to control ($P = 0.01$). However, when IRAP was administered 1 h after injury, expressions of MMP-3, IL-1β and TNF-α were significantly lower than the injury group ($P = 0.009$, 0.003, and 0.003, respectively). Expressions of miR-125b and miR-27b were also significantly increased in meniscus after injury and treatment as compared to control ($P = 0.005$ and 0.001, respectively) and as compared to injury group ($P = 0.002$ and $P < 0.001$, respectively).

Discussion

The *ex-vivo* intact joint impact injury model developed in this study presents a method to examine the acute phase of impact injury and can be used to investigate the efficacy of potential treatments. A detailed understanding of the events that occur during the acute phase injury in cartilage can provide great insight

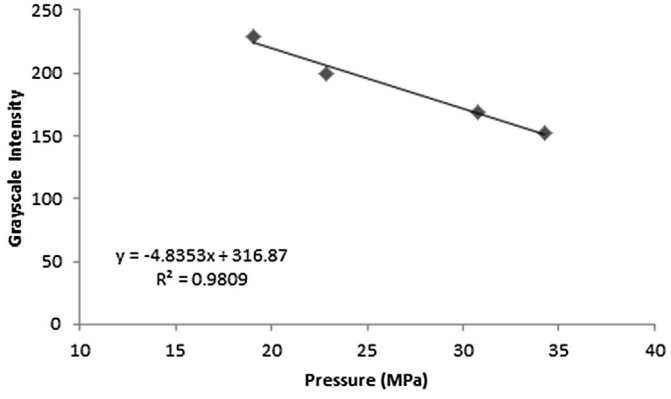

Fig. 1. Calibration curve showing a linear relationship between grayscale image intensity and applied pressure.

or help choose suitable time points, targets, and methods of interventional treatment to prevent or delay the progression of PTOA. The results of this study support the hypothesis by demonstrating that early intervention with IRAP during the acute phase of cartilage injury significantly up-regulated expression of miRNA and inhibited up-regulation of their target mRNA induced by impact. This is the first study to report differential miRNA expressions in cartilage after impact injury and the effects of IRAP intra-articular treatment on miRNA expression.

In effort to determine the mechanism of PTOA development, researchers have developed various *in-vitro* and *in-vivo* models of impact injury to cartilage. Cartilage explant models[30–32] have the ability to transduce the mechanical forces associated with impact injuries; however, they are maintained in non-physiological culture conditions with high amounts of nutrients and oxygen as compared to the avascular, hypoxic environment within the knee joint. Rundell *et al.* determined that the true cartilage explant response to mechanical loading is altered by equilibration in standard culture medium[33]. While the *in-vivo* impact injury model[20,34] remains the "gold-standard" for investigating the true response of cartilage after injury, the *ex-vivo* intact joint injury model utilized in this study provides a physiological acute injury response with minimal disruption of native tissue environment. The *ex-vivo* injury model has proven consistent with *in-vivo* models of acute cartilage injury that reported peak IL-1β expression within 4 h of injury in an *in-vivo* murine model[34].

In normal joint loading *in vivo*, the force is distributed 70% to medial condyle and 30% to the lateral condyle[35]. However, even though the forces are unequally distributed, the pressures remain similar due to the larger contact area and cartilage thickness of the medial condyle[36]. Based on the Fujifilm data, it was found that both femoral condyles were injured simultaneously while similar maximum contact stresses on the condyles were observed, showing that a physiological distribution of stresses on the cartilage was effectively created in the *ex-vivo* injury model using an intact joint. Furthermore, the contact stresses, greater than 20 MPa, covered a total combined area of 23.5 ± 1.9 mm^2 of cartilage. The impact forces applied and pressures recorded are within the range of deleterious cartilage impact as pressures of 20 MPa or greater have been reported to lead to critical cell death, collagen matrix rupture, and eventual cartilage degeneration in several long term *in-vitro* and *in-vivo* studies[20–23]. It can be inferred that the physiologically relevant acute injury response of cartilage is accurately represented in this model, as confirmed by viability staining of injured area.

Aggrecanases are integral components of cartilage homeostasis and matrix remodeling; however, after traumatic impact injury, production of these enzymes becomes significantly up-regulated, resulting in a compromised matrix structure with reduced mechanical stability[32]. Loss of aggrecan in the cartilage matrix is observed as one of the earliest changes in cartilage degeneration and OA progression[37] with changes reported as early as 1 day after injury[38]. Down-regulation of miR-125b and miR-140 has been directly linked to elevated expression levels of ADAMTS-4 and ADAMTS-5, respectively[8,9]. At 8 h post-injury, miR-140 and miR-125b expressions were significantly down-regulated, indicating that at 8 h after injury, cartilage has reduced inhibition of aggrecanases. However, IRAP intra-articularly injected 1 h after impact injury inhibits catabolic aggrecanase expression with increased expressions of corresponding regulatory miRNAs. Therefore, IRAP treatment may inhibit the production of aggrecanase production through the up-regulation of miR-140 and miR-125b, thereby preventing potential cartilage degradation.

MMP-3 cleaves aggrecan and activates pro-MMP-13, the most potent collagen-degrading enzyme[11,39]. MMP-3 expression was significantly up-regulated at 8 h after injury. In injured cartilage

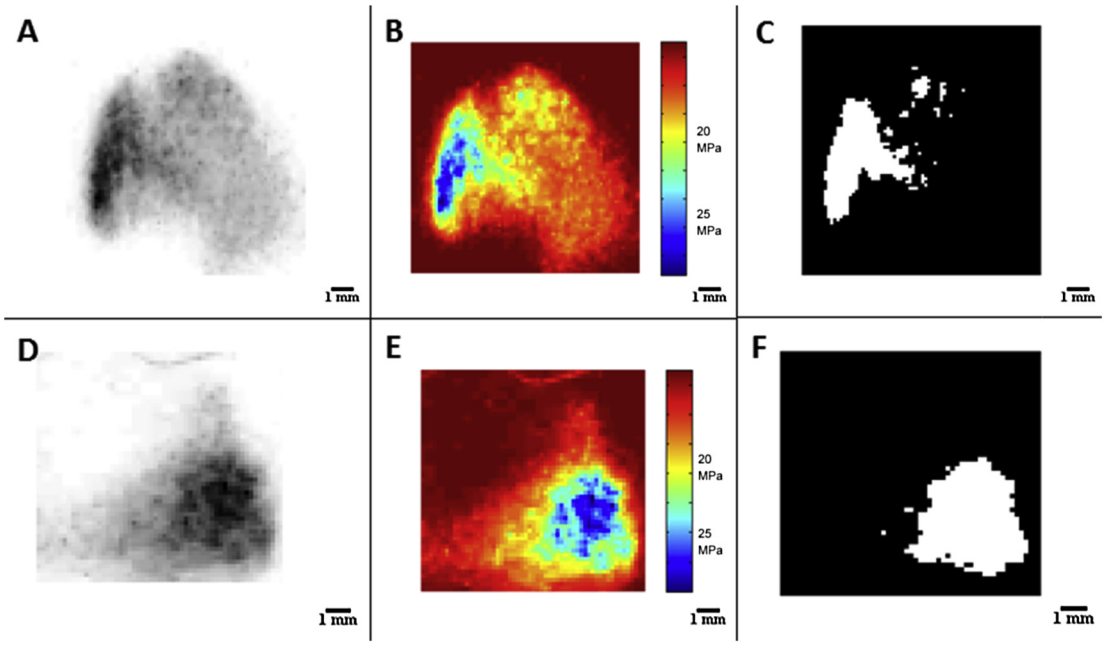

Fig. 2. Example analysis of Fujifilm: typical medial condyle stain (A–C) and typical lateral condyle stain (D–F). A&D. Adjusted grayscale stain of digital raw stain (600 ppi) with averaged intensities calculated over areas 4 × 4 pixel areas and applied pressure distribution map. B&E. Colormap of pressures. C&F. Region identified in white with >20 MPa applied pressure.

with IRAP treatment, MMP-3 expression is no longer significantly up-regulated as compared to controls. Furthermore, as the direct regulator of MMP-13[10], miR-27b expression was significantly up-regulated after IRAP treatment, similarly indicating increased inhibition of collagenase. The expressions of these degenerative enzymes in the acute phase of injury and their subsequent inhibition after IRAP treatment greatly support the potential of early intervention to prevent cartilage degeneration.

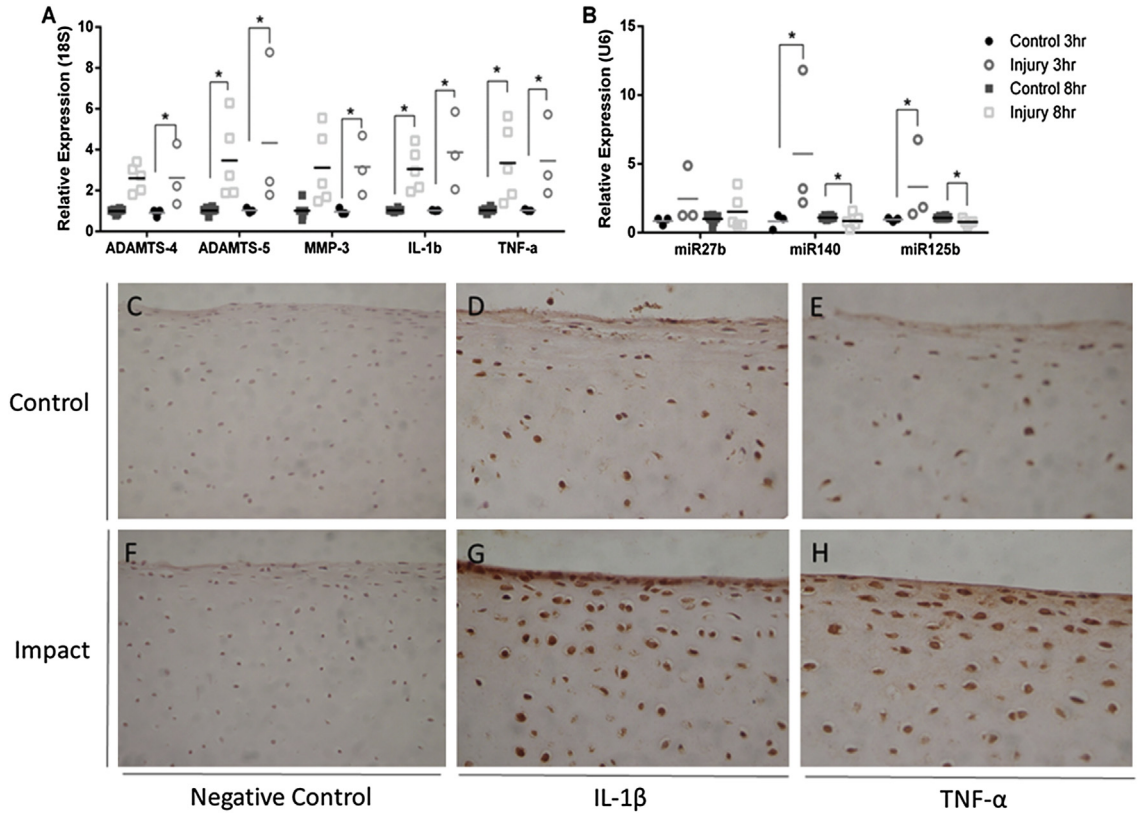

Fig. 3. (A,B) Relative expressions of inflammatory cytokines and catabolic enzymes (A) and expressions of miR-125b, miR-140, and miR-27b (B) at 3 and 8 h after impact injury. *indicates significance $P < 0.05$. Refer to text for exact P-value. (C–H) Immunohistochemical assessments of control and injured cartilage for expressions of IL-1β (D,G) and TNF-α (E,H). Negative control sections for control (B) and injured (F) cartilage showed negligible staining. Representative samples are shown. Original magnification × 200.

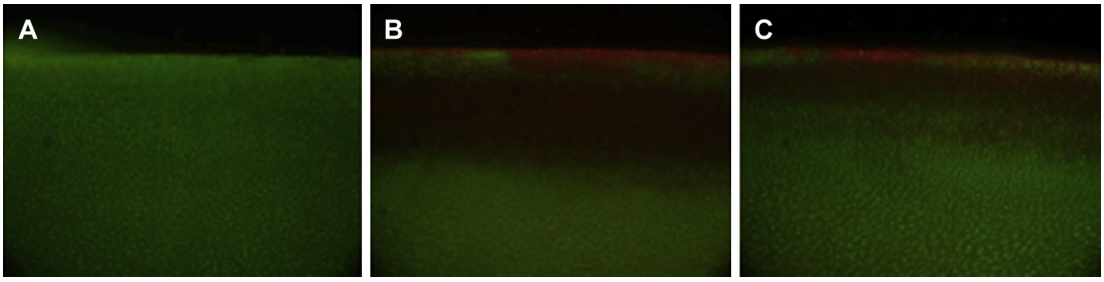

Fig. 4. Representative cell viability staining of cartilage at 8 h post-injury and IRAP treatment (10×) A. Control; B. Injury, No Treatment; C. Injury, IRAP treatment. Live cells (green), dead cells (red).

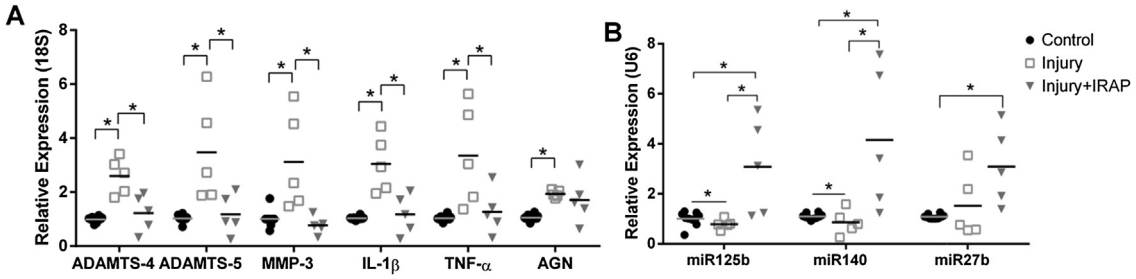

Fig. 5. Relative expressions of inflammatory cytokines, catabolic enzymes, and aggrecan (A) and expressions of miR-125b, miR-140, and miR-27b (B) in control, injured, and treated cartilage at 8 h after impact injury. *indicates significance $P < 0.05$. Refer to text for exact *P*-value.

The results of this study can be compared to inhibitor studies of other downstream targets in the IL-1 pathway, such as nuclear factor (NF)-κβ, protein 38 MAP kinase (p38 MAPK), and c-Jun N-terminal kinase (JNK) pathway. Inhibition of the NF-κβ, p38 MAPK, and JNK pathways significantly suppressed IL-1-induced aggrecanase expression and activity in chondrocytes[40]. Additionally, in IL-1 stimulated OA chondrocytes, NF-κβ inhibitors significantly increased miR-27b expression[10]. Nonetheless, the evidence of inhibition of catabolic gene expression in acutely injured cartilage after IRAP treatment should be considered preliminary; therefore, any findings need to be confirmed and expanded upon in longitudinal *in-vivo* studies.

Inflammation in the cartilage and knee joint, driven by IL-1β and TNF-α[5,41], plays a crucial role in the development of OA. High mechanical stress on chondrocytes activates the IL-1 pathway and results in the secretion of IL-1β[14,41]. It is well understood that exogenous IL-1β secreted by surrounding knee tissues incites a cascading effect on the catabolic responses of cartilage[5]. In this study, IL-1β and TNF-α are significantly up-regulated at 3 h and remain up-regulated at 8 h after impact injury as shown by gene expression analysis and immunohistochemistry. Concurrently, mechanical injury incites transient up-regulation of miR-125b, miR-140, and ADAMTS-5 at 3 h post-impact. However, at 8 h, secreted IL-1β binds to the IL-1 receptor, which incites the IL-1 catabolic pathway, and then down-regulates miR-140 and miR-125b. As a result, elevated aggrecanase and inflammatory cytokine expressions remain at 8 h post-injury. Nevertheless, after intra-articular injection of IRAP given 1 h after impact, IL-1β and TNF-α were no longer up-regulated at 8 h. Administration of IRAP serves to antagonize the catabolic cascade (Fig. 7) by preventing IL-1β from binding to the IL-1 receptor on the cell membrane, and inhibits the paracrine and autocrine effects of IL-1β secreted due to mechanical loading[12]. Therefore, expressions of miR-140 and miR-125b remain elevated and expressions of aggrecanases and inflammatory cytokines are down-regulated.

At 8 h, aggrecan expression was significantly increased by impact. Previous studies have reported transient up-regulation of aggrecan during the early phase of injurious compression[42–44]. However, consistent with previous studies in meniscus and cartilage tissues[13,14], we found no significant differences in aggrecan or collagen II expressions between injury and treatment groups at 8 h. Therefore, the transient up-regulation in aggrecan expression was

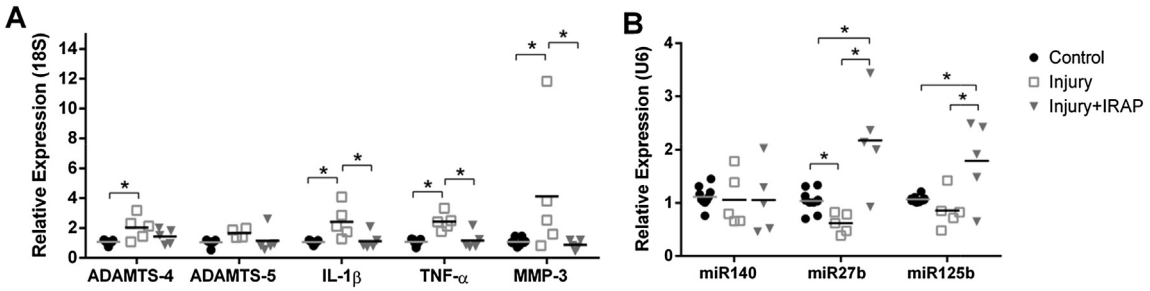

Fig. 6. Relative expressions of inflammatory cytokines and catabolic enzymes (A) and expressions of miR-125b, miR-140, and miR-27b (B) in control, injured, and treated meniscus at 8 h after impact injury. *indicates significance $P < 0.05$. Refer to text for exact *P*-value.

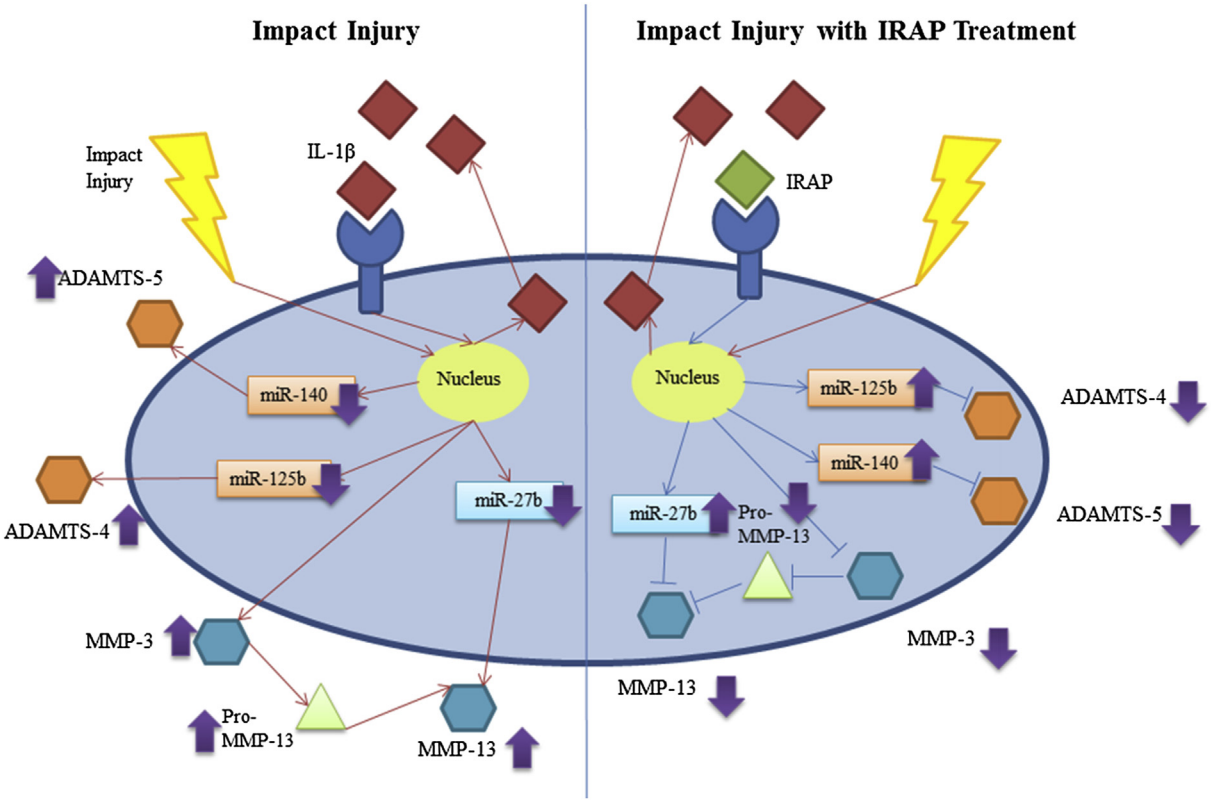

Fig. 7. A proposed mechanism of IRAP treatment. Impact injury causes up-regulation of IL-1β expression and secretion, which binds to the IL-1 receptor, incites the IL-1 catabolic pathway, and results in down-regulated miR-140 and miR-125b expressions and up-regulated aggrecanase expressions. IRAP prevents IL-1β from binding to the IL-1 receptor on the cell membrane and inhibits the paracrine and autocrine effects of IL-1β secreted. Therefore, expressions of miR-140 and miR-125b remain elevated due to mechanical loading and expressions of aggrecanases and inflammatory cytokines are down-regulated. Up-regulation or down-regulation of a specific gene is represented by an upward or downward arrow, respectively. All listed targets have been validated. The miRNA targets listed include aggrecanase-1 (ADAMTS-4), aggrecanase-2 (ADAMTS-5), and collagenase-3 (MMP-13).

caused by mechanical injury to cartilage and not by increased IL-1β expression.

In the *ex-vivo* injury model, the meniscus was also injured in a physiologically compressive manner. Impact injury caused significant down-regulation of miR-27b and significant up-regulation of catabolic enzymes and inflammatory cytokines in meniscus. After injury and IRAP treatment, miR-125b and -27b were significantly up-regulated, and catabolic enzyme and inflammatory cytokine expressions in meniscus were significantly reduced through a mechanism comparable to cartilage. Although articular cartilage and meniscus are primarily made of chondrocytes, the two tissues distribute loads differently under impact. Different distributed loads, along with inherent genetic and material properties[45], could the differences in the miRNA responses after impact from cartilage and meniscus tissues.

Upon the comparison of the control, injury, and treatment groups, a significant decrease in cell viability was seen between control and injury groups. The observed decrease in viability and region of cell death is consistent with *in-vitro* explant models[19,23] and clinical studies[46]. While most of the superficial layer chondrocytes would have died by necrosis from the excessive strain induced by impact[47], we expect that IRAP treatment administered 1 h after impact may have prevented IL-1β from inciting cellular apoptosis by blocking the IL-1 receptors[48,49].

The rationale for administering treatment at 1 h after injury was based on the need to demonstrate proof of concept but probably not mimic a clinically relevant situation in the health care system where access to care is limited. The model utilized in this study presents the ability to monitor the acute events after injury while maintaining the physiological environment of the knee joint tissues. A possible limitation of this model is the inability to perform long-term studies. While cartilage tissue remains viable for over 24 h after donor death[50], meniscus tissue viability could decrease without renewed blood supply.

In summary, this study demonstrates that IRAP treatment administered during acute phase of articular cartilage impact injury increases expression levels of miR-140, miR-125b, and miR-27b with significant down-regulation of inflammatory cytokines and matrix-degrading enzymes. The aberrant regulation of degenerative enzymes and inflammatory cytokines in cartilage, along with a decrease in chondrocyte viability, after impact injury increases the risk of PTOA development. Clinically, these findings support the potential of IRAP treatment as an early intervention strategy for the prevention of cartilage degeneration after impact injury.

Author contributions

Design of the study; AG, CH, LK. Performed the experiments; AG, CH, TR. Analyzed the results; AG, TR, CH, LK. Drafting of the manuscript; AG, TR, CH. Manuscript revision; CH, LK. All authors approved final version of manuscript for submission.

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Conflict of interest

The authors report no conflict of interest with this study.

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